REMARKS

In the Office Action dated September 20, 2005, claims 49, 54-59, 64-69, 74-79 and 84-88 were examined with the result that claims 59, 64-69, 74-79 and 84-88 were all allowed, and claims 49 and 54-58 were rejected. In response, Applicant submits two exhibits and the following comments. In view of the exhibits and comments, reconsideration of this application is requested.

At the bottom of page 2 of the Office Action, the Examiner indicated that the comparative data previously discussed in the Amendment of July 21, 2005 clearly show the differences between 2MD (the closest prior art compound) and the presently claimed 26,27-dihomo compound. The Examiner indicated that Applicants have clearly demonstrated that the activities of these two compounds are not the same and the results were not expected, i.e. the presently claimed 26,27-dihomo compound showed significantly greater intestinal calcium transport activity than 2MD. However, the Examiner indicated on page 3 of the Office Action that it was not clear what advantage the 26,27-dihomo compound had over 2MD in the claimed methods since the claims 49 and 54-58 are drawn to a method of treating a cancerous disease with the 26,27-dihomo compound. In response, Applicant has the following comments.

Applicant encloses two pages of data concerning the 26,27-dihomo compound and the 2MD compound. The first exhibit is page 4668 from the Journal of Medicinal Chemistry, 1998, Vol. 41, No. 23, which illustrates in the highlighted area the HL-60 cell differentiation response of the compound 2MD. The second exhibit is a copy of page 252 from a paper published in Steroids, 2002, Vol. 67 which illustrates in the highlighted area the HL-60 cell differentiation response of the 26,27-dihomo compound. The data reported are the ED_{50} values for the two compounds. The ED_{50} values are derived from dose response curves and represent the analog concentration required for 50% displacement of the radio labeled 1α,25-dihydroxyvitamin D_3 from the receptor protein. (See the notes beneath the tables in both exhibits).

The ED_{50} reported for 2MD is 1.5×10^{-10} while the ED_{50} value for the 26,27-dihomo compound is 2.6×10^{-11}. The data show that the 26,27-dihomo compound is approximately ten times more effective than 2MD at cellular differentiation, and therefore, would be unexpectedly better at treating such diseases as cancer because it

causes cellular differentiation at a much lower dose. Said another way, at the same dosage, the 26,27-dihomo compound is about ten times more effective than 2MD at cellular differentiation. HL-60 cellular differentiation activity has long been accepted as a model for potential effectiveness against cancerous diseases such as leukemia, colon cancer, breast cancer and prostate cancer as claimed by Applicant. This link or relationship between cellular differentiation and these four cancer therapies was adequately discussed at pages 10 and 11 of Applicant's prior Amendment dated July 21, 2005. Applicant refers the Examiner to those comments.

In the Office Action, the Examiner rejected claims 49 and 54-58 under the Doctrine of Obviousness Type Double Patenting as the Examiner was unclear of the advantage of using the dihomo compound over the compound 2MD. However, in view of the above comments and enclosed exhibits, Applicant believes it has adequately described the advantages of the 26,27-dihomo compound over 2MD. It is clear that although the two compounds are structurally similar, and therefore would be expected to have similar properties, the above arguments and enclosed exhibits clearly show that 26,27-dihomo would be more effective against cancerous diseases than 2MD, and this activity is unexpected based upon their structural similarity. Accordingly, Applicant requests the Examiner withdraw the obviousness type double patenting rejection.

An effort has been made to place this application in condition for allowance and such action is earnestly requested.

Respectfully submitted,

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Table 1. VDR Binding Properties[a] and HL-60 Differentiating Activities[b] of 2-Substituted Analogues of 1α,25-Dihydroxy-19-norvitamin D_3 and Their 20S-Isomers

compound	compd no.	VDR binding		HL-60 differentiation	
		ED_{50} (M)	binding ratio	ED_{50} (M)	activity ratio
$1\alpha,25\text{-}(OH)_2D_3$	1	8.0×10^{-11}	1	4.0×10^{-9}	1
2-methylene-19-nor-$1\alpha,25\text{-}(OH)_2D_3$	6a	1.2×10^{-10}	1.5	4.2×10^{-9}	1
2-methylene-19-nor-(20S)-$1\alpha,25\text{-}(OH)_2D_3$	6b	1.0×10^{-10}	1.3	1.6×10^{-10}	0.04
$1\alpha,25\text{-}(OH)_2D_3$	1	9.0×10^{-11}	1	4.0×10^{-9}	1
2α-methyl-19-nor-$1\alpha,25\text{-}(OH)_2D_3$	7a	4.2×10^{-10}	4.6	8.0×10^{-11}	0.02
2α-methyl-19-nor-(20S)-$1\alpha,25\text{-}(OH)_2D_3$	7b	4.0×10^{-10}	4.4	7.0×10^{-11}	0.02
2β-methyl-19-nor-$1\alpha,25\text{-}(OH)_2D_3$	8a	3.5×10^{-9}	39	8.0×10^{-9}	2.0
2β-methyl-19-nor-(20S)-$1\alpha,25\text{-}(OH)_2D_3$	8b	5.0×10^{-10}	5.5	7.0×10^{-10}	0.17
$1\alpha,25\text{-}(OH)_2D_3$	1	6.0×10^{-11}	1	4.0×10^{-9}	1
2α-(hydroxymethyl)-19-nor-$1\alpha,25\text{-}(OH)_2D_3$	9a	8.0×10^{-10}	13	2.0×10^{-8}	5.0
2α-(hydroxymethyl)-19-nor-(20S)-$1\alpha,25\text{-}(OH)_2D_3$	9b	8.0×10^{-11}	1.3	2.0×10^{-9}	0.5
2β-(hydroxymethyl)-19-nor-$1\alpha,25\text{-}(OH)_2D_3$	10a	7.0×10^{-9}	117	1.0×10^{-6}	25
2β-(hydroxymethyl)-19-nor-(20S)-$1\alpha,25\text{-}(OH)_2D_3$	10b	5.0×10^{-10}	8.3	4.5×10^{-8}	1.1

[a] Competitive binding of $1\alpha,25\text{-}(OH)_2D_3$ (1) and the synthesized vitamin D analogues to the porcine intestinal vitamin D receptor. The experiments were carried out in triplicate on two different occasions. The ED_{50} values are derived from dose–response curves and represent the analogue concentration required for 50% displacement of the radiolabeled $1\alpha,25\text{-}(OH)_2D_3$ from the receptor protein. Binding ratio is the ratio of the analogue average ED_{50} to the ED_{50} for $1\alpha,25\text{-}(OH)_2D_3$. [b] Induction of differentiation of HL-60 promyelocytes to monocytes by $1\alpha,25\text{-}(OH)_2D_3$ (1) and the synthesized vitamin D analogues. Differentiation state was determined by measuring the percentage of cells reducing nitro blue tetrazolium (NBT). The experiment was repeated three times. The ED_{50} values are derived from dose–response curves and represent the analogue concentration capable of inducing 50% maturation. Differentiation activity ratio is the ratio of the analogue average ED_{50} to the ED_{50} for $1\alpha,25\text{-}(OH)_2D_3$.

2-methyl-substituted 19-norvitamins 7a,b and 8b were only 4–5 times less active than $1\alpha,25\text{-}(OH)_2D_3$, while the 2β-methyl isomer in the 20*R*-series (8a) was 39-fold less effective. The 2α-(hydroxymethyl)vitamin D analogue with the "unnatural" configuration at C-20 (9b) was almost equivalent to the hormone 1 with respect to receptor binding, and the isomeric 10b proved to be less potent (6–8 times) than these compounds. The corresponding 2α-hydroxymethyl analogue possessing the "natural" 20*R*-configuration (9a) was 10-fold less effective than the 20*S*-compound 9b; whereas the 2β-isomer 10a was 90 times less potent. The foregoing results of the competitive binding analysis show that vitamins with the axial orientation of the 1α-hydroxy group exhibit a significantly enhanced affinity for the receptor. Both isomeric 1α,19,25-trihydroxy-10,19-dihydrovitamin D_3 compounds 13 and 14 were 1000 times less potent than the natural hormone 1 in displacement of the radiolabeled $1\alpha,25\text{-}(OH)_2D_3$ from the receptor protein (data not shown).

Attention is drawn to the fact that both 2-methylene-19-norvitamins 6a,b, when tested in vivo in rats, exhibited an extremely high ability to mobilize calcium from bone, while having no intestinal calcium transport activity (Table 2). It was not surprising that the 1α,-19,25-trihydroxy-10,19-dihydrovitamin D_3 compounds 13 and 14 were devoid of biological activity (data not shown), in view of our previous results indicating low potency of the parent 1α,25-dihydroxy-10,19-dihydrovitamin D_3 isomers.[12] However, vitamins possessing a hydroxymethyl substituent at C-2 turned out to be inactive, including those in the 20*S*-series (9b, 10b). Thus, on the basis of the results described above, it was impossible to draw any conclusions regarding the conformation–activity relationship. On the contrary, the in vivo biological testing of 2-methyl-substituted 19-nor-$1\alpha,25\text{-}(OH)_2D_3$ analogues appeared to be much more interesting and informative. Table 2 shows that a 260-pmol dose of 2α-methyl vitamin 7a is equal to or only slightly less effective on intestinal calcium transport and bone calcium mobilization than hormone 1. The overall calcemic activity of its analogue 7b from the 20*S*-series proved to be appreciably higher than that of $1\alpha,25\text{-}(OH)_2D_3$. However, no elevation of serum calcium or bone calcium mobilization was found for any of the 2β-methyl analogues 8a,b at this dose level. The foregoing activity of the vitamin D analogues strongly depends on the conformation of the A-ring. However, in any case, these results do not support the suggestion that the equatorially favored 1α-hydroxyl is required for calcemic activity, because analogues with such an orientation of the 1α-OH group show reduced biopotency in both intestine and bone. Thus, the results indicate a much greater biological activity of the vitamins possessing the axial 1α-hydroxy substituent.

In the next assay, the cellular activity of the synthesized compounds was established by studying their ability to induce differentiation of human promyelocytes HL-60 cells into monocytes. Interestingly, the transposition of the A-ring exocyclic methylene group to C-2 did not change the cellular differentiation ability of the analogue 6a with respect to the parent hormone 1 (Table 1). It was found that, with an exception of an equally active 10b, all of the synthesized vitamin D analogues with the "unnatural" 20*S*-configuration were more potent than $1\alpha,25\text{-}(OH)_2D_3$. Moreover, the same relationship between cellular activity and conformation of the vitamin D compounds was established as in the case of receptor binding analysis and in vivo studies: i.e., 2α-substituted vitamin D analogues were considerably more active than their 2β-substituted counterparts with the equatorially oriented 1α-hydroxy group. Thus, 2α-methyl vitamins 7a,b proved to be 100 and 10 times, respectively, more active than their corresponding 2β-isomers 8a,b in the cultures of HL-60 in vitro, whereas in the case of 2-hydroxymethyl derivatives (9a,b versus 10a,b) these differences were smaller. Since vitamins with a 2β-methyl substituent (8a,b) and both 2-hydroxymethyl analogues in the 20*S*-series (9b, 10b) have selective activity profiles combining high potency in cellular differentiation and lack of calcemic activity, such compounds are potentially useful as therapeutic

Table 1
VDR binding properties[a] and HL-60 differentiating activities[b] of 2-substituted side-chain modified (20S)-1α,25-dihydroxy-19-norvitamin D_3 analogs

Compound	Compd. no.	VDR Binding		HL-60 Differentiation	
		ED_{50} (M)	Binding ratio	ED_{50} (M)	Activity ratio
$1\alpha,25\text{-}(OH)_2D_3$	1a	8.7×10^{-10}	1	4.0×10^{-9}	1
2-Methylene-26,27-dihomo-19-nor-(20S)-1α,25-$(OH)_2D_3$	7	4.3×10^{-9}	0.20	2.6×10^{-11}	154
2α-Methyl-26,27-dihomo-19-nor-(20S)-1α,25-$(OH)_2D_3$	8	3.1×10^{-9}	0.28	6.0×10^{-11}	67
2β-Methyl-26,27-dihomo-19-nor-(20S)-1α,25-$(OH)_2D_3$	9	4.8×10^{-9}	0.18	1.1×10^{-10}	36
2-Methylene-26,27-dimethylene-19-nor-(20S)-1α,25-$(OH)_2D_3$	11	2.7×10^{-9}	0.32	3.6×10^{-11}	111
2-Methylene-26,27-dimethylene-24-dehydro-19-nor-(20S)-1α-OH-D_3	12	2.9×10^{-8}	0.03	4.1×10^{-9}	0.98
2-Methylene-26,27-dimethylene-25-methoxy-19-nor-(20S)-1α-OH-D_3	13	1.5×10^{-8}	0.06	4.3×10^{-9}	0.93
2α-Methyl-26,27-dimethylene-19-nor-(20S)-1α,25-$(OH)_2D_3$	14	3.5×10^{-9}	0.25	4.4×10^{-11}	91
2β-Methyl-26,27-dimethylene-19-nor-(20S)-1α,25-$(OH)_2D_3$	15	2.3×10^{-9}	0.38	3.2×10^{-10}	12.5

[a] Competitive binding of $1\alpha,25\text{-}(OH)_2D_3$ and the synthesized vitamin D analogs to the porcine intestinal vitamin D receptor. The experiments were carried out in triplicate on two different occasions. The ED_{50} values are derived from dose-response curves and represent the analog concentration required for 50% displacement of the radiolabeled $1\alpha,25\text{-}(OH)_2D_3$ from the receptor protein. Binding ratio is the ratio of the ED_{50} for $1\alpha,25\text{-}(OH)_2D_3$ to the analog average ED_{50}.

[b] Induction of differentiation of HL-60 promyelocytes to monocytes by $1\alpha,25\text{-}(OH)_2D_3$ and the synthesized vitamin D analogs. Differentiation state was determined by measuring the percentage of cells reducing nitro blue tetrazolium (NBT). The experiment was repeated three times. The values ED_{50} are derived from dose-response curves and represent the analog concentration capable of inducing 50% maturation. Differentiation activity ratio is the ratio of the ED_{50} for $1\alpha,25\text{-}(OH)_2D_3$ to the analog average ED_{50}.

They were then switched to the reduced calcium diet (0.02% Ca) for an additional 2 weeks. These animals have no detectable levels of 25-OH-D_3 or $1\alpha,25\text{-}(OH)_2D_3$ in their plasma as measured by methods described previously [32]. For this first experiment, the indicated rats received a single i.v. dose of the indicated compound in 0.05 ml of ethanol (data not shown). In the other experiment, the rats were given the indicated doses of compounds in 0.1 ml of (95:5) 1,2-propanediol/ethanol by intraperitoneal (i.p.) injection each day for 7 days. In the first experiment, the rats were euthanized at various times after the dose (data not shown). In the second experiment, they were sacrificed 24 h after the last dose. The rats were sacrificed under ether anesthesia by decapitation, their blood and intestines were collected and used immediately to determine calcium transport activity and serum calcium concentration. Calcium was measured in the presence of 0.1% lanthanum chloride by means of a Perkin-Elmer atomic absorption spectrometer Model 3110. Intestinal calcium transport was determined by the everted intestinal sac method using the proximal 10 cm of intestine as described earlier [31]. Statistical analysis was by the Student's *t*-test [33]. Intestinal calcium transport is expressed as serosal:mucosal ratio of calcium in the sac to the calcium in the final incubation medium or S/M. Bone calcium mobilization represents the rise in serum calcium of the rats maintained on a very low calcium diet. In that measurement, the rise in serum calcium must arise from bone and hence is a determination of bone calcium mobilization.

2.9. Measurement of cellular differentiation

Human leukemia HL-60 cells, originally obtained from ATTC, were plated at 2×10^5 cells per plate, incubated in Eagle's modified medium as described previously [34]. The compounds tested were added in the indicated concentrations in 0.05 ml of ethanol so that the ethanol concentration never exceeded 1%. The incubation was carried out for 4 days and at the end of the 4th day, superoxide production was measured by nitro blue tetrazolium (NBT) reduction. The number of cells containing intracellular black-blue formazan deposits was determined by light microscopy using a hemacytometer. At least 200 cells were counted in duplicate per determination. Percentage differentiation represents percentage cells providing NBT reduction appearance. The results were plotted on semilog paper, and relative differentiation activities of the analogs were determined by comparison of the compound concentrations capable of inducing 50% maturation according to the assay. This method is described in detail elsewhere [34]. The experiment was repeated 3 times and the results are reported as the mean ± SD.

2.10. Measurement of binding to the porcine intestinal vitamin D receptor

Porcine intestinal nuclear extract was prepared as described earlier [35].